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14. ABSTRACT The main goal of this study is to overcome docetaxel (DTX) resistance in prostate cancer cells by combined delivery of eIF4E siRNA and DTX using dendrimer as a nanocarrier. To this end the objective of this study is to prepare, characterize and test the feasibility of the combination delivery approach <i>in-vitro</i> in DTX sensitive and resistant prostate cancer cells. During the last evaluation period we prepared and characterized the dendrimer-DTX conjugate and dendrimer-siRNA complex. During this evaluation period our goal was to optimize the delivery system, develop the DTX resistant prostate cancer cells and test the in-vitro efficacy of the multifunctional delivery system. The DTX resistant prostate cancer cells were developed by treating PC3 cells with sub-therapeutic concentration of DTX for 9 months and it was found to overexpress eIF4E. The multifunctional therapeutic system was able to overcome the resistance in PC3 cells and lowered the IC ₅₀ value in drug resistant PC3 cells. The siRNA reduced the levels of eIF4E and Myc in PC3 cells. Overall the outcomes from this study demonstrate the feasibility of developing a therapeutic strategy for drug resistant prostate cancer. Our future studies will focus on further optimizing the delivery system and testing the efficacy in-vivo in prostate cancer mouse model.					
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Introduction

Most prostate cancer (PC) patients progress to advanced castration-refractory prostate cancer (CRPC) within a few years of androgen ablation therapy¹. Although docetaxel (DTX) has shown improved survival in CRPC patients, resistance to treatment severely limits patient survival². The treatment induced overexpression of cell survival and anti-apoptotic proteins leads to drug resistance. In this regard eukaryotic translation initiation factor 4E (eIF4E) plays a critical role in regulating the translation of mRNAs that encode several proteins involved in cell growth and survival³. Our goal is to overcome this drug resistance by combined delivery of eIF4E siRNA and DTX using dendrimer as a nanocarrier. To this end the specific aims of this study are i) to test whether a combination of eIF4E siRNA and DTX results in increased activity in PC cells, ii) to prepare and characterize dendrimer-DTX-siRNA nanotherapeutic system, iii) to test the efficacy of nano-therapeutic system in PC3 and PC3dR cells. During the last evaluation period we developed and characterized DTX-dendrimer conjugate and siRNA-dendrimer complex. However our attempts to develop drug resistant PC3 cells were not successful. During this evaluation period we procured DTX-resistant PC3 cells from a leading prostate cancer research group in Canada⁴. We were able to successfully develop the resistant cells by treating PC3 cells with sub-therapeutic DTX concentrations for 9 months. The eIF4E levels in drug resistant PC3 cells were increased by 2 fold compared to drug sensitive PC3 cells. We also successfully developed the multifunctional therapeutic system by combining DTX and siRNA in dendrimer based nanocarrier. When combined with siRNA, the IC₅₀ value for DTX was significantly reduced in drug-resistant PC3 cells. The details are reported below.

Body

Specific Aim 1: To Test the activity of eIF4E siRNA and DTX in PC cells

During the last evaluation period we tested the combination delivery approach in drug sensitive PC3 cells. It was found that the combination of siRNA-liposomes and DTX produced slightly higher cell death compared to individual treatment groups. Given the PC-3 cells have normal levels of eIF4E, the results were not surprising. Our initial attempts to develop drug resistant PC3 cells were unsuccessful. During this evaluation period our main goal was to develop drug resistant PC3 cells. To this end we obtained drug resistant PC3 cells from a leading prostate cancer research group in Canada. Their protocol was followed in developing the DTX resistant prostate cancer cells (PC3dR).

DTX resistant prostate cancer cells were developed by treating PC3 cells with sub-therapeutic concentrations of DTX (5, or 10 nM). The PC3dR 10nM docetaxel cell line was used for all subsequent experiments. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum (FBS) and 1% streptomycin. The PC-3 cells were serially treated with 1 week of docetaxel chemotherapy at dose of 10 nM ('on cycle'), followed by 1 week with no treatment ('off cycle'). PC-3dR cell lines were developed after using eighteen 'on' and eighteen 'off' cycles for a total of 36 weeks. The development of drug resistance was determined using MTT assay. The IC₅₀ value of DTX for PC3dR (~500 nM) cells was almost 2.5 times higher than the normal PC3 cells (100 nM). Western blot analysis was performed to determine the eIF4E levels. The eIF4E levels were elevated PC3-dR by 2 fold compared to the drug sensitive PC3 cells (pls refer Fig . 6 presented later in this report).

Specific Aim 2: Prepare and characterize dendrimer-DTX-siRNA conjugate

During the last evaluation period, we developed a synthetic method for the preparation of DTX- dendrimer conjugate. We found that hemisuccinate-DTX is a suitable DTX derivative for conjugating to the primary amine groups in PAMAM dendrimer. The conjugate was characterized by UV-spectroscopy, FTIR, NMR and MALDI-TOF. It was found that the 9 molecules of DTX was conjugated to dendrimer. Further we also developed siRNA-dendrimer complex using various ratios of siRNA and dendrimer. During this evaluation period our goal was to further optimize the DTX and siRNA molecules in dendrimer and test its in-vitro release.

We further performed the controlled conjugation of the DTX to the PAMAM dendrimer using the synthetic method developed in the previous evaluation period. The number of DTX molecules was optimized by varying the ratio of DTX:dendrimer (Table 1). The conjugates were characterized using FTIR and Proton NMR and MALDI TOF⁵. The MALDI-TOF was performed using α -cyano-4-hydroxycinnamic acid as the matrix (identified as a suitable matrix during the last evaluation period). As shown in Table 1 and Fig 1, three (MW 16390.017 figure) and seven

(MW 19337.609 figure) DTX molecules were conjugated to dendrimer when 1:4 and 1:2 molar ratio of DTX:dendrimer were used respectively.

Table 1: Characterization of DTX-dendrimer conjugates

Dendrimer/Dendrimer Conjugate	Molar ratio of DTX-SUC to NH ₂ groups on dendrimer surface used for conjugation	Molecular Weight (Da) by MALDI-TOF	Approximate no. of DTX (Mwt 807) molecules conjugated per dendrimer
G4 PAMAM	-	13653.77	-
G4 PAMAM- DTX (16)	16 : 64 (1:4)	16390.017	3.39
G4 PAMAM- DTX (32)	32 : 64 (1:2)	19337.609	7.05
G4 PAMAM- DTX (64)	64:64 (1:1)	22495.481	10.96

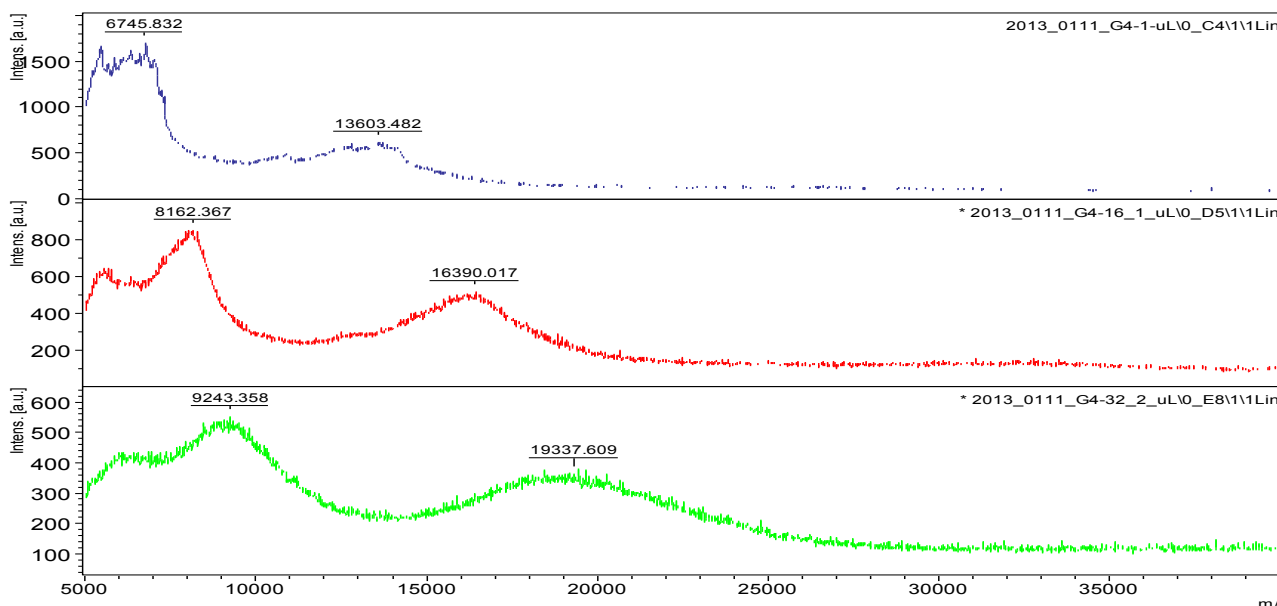
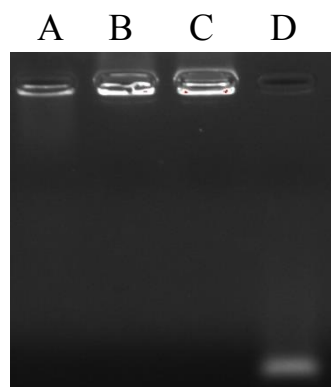


Fig 1 : MALDI-TOF results of controlled conjugation of the DTX to PAMAM dendrimer (A) PAMAM (B) DTX-PAMAM (1:4) (C) DTX-PAMAM (1:2).

The above optimized DTX-dendrimer conjugate was used to complex eIF4E siRNA. Briefly, DTX-PAMAM and siRNA was incubated at different N/P ratios (5:1, 10:1, 20:1) in RNase free water for about 30 minutes. The formation of complex was confirmed using agarose gel electrophoresis. Agarose gel electrophoresis was performed using TBE buffer at 100 mV for 30 minutes. Briefly, 2% agarose was dissolved in 1x TBE buffer with addition of 0.5 µg/mL solution of ethidium bromide. Each well was loaded with 10 µL of the dendrimer-siRNA complex (with different N/P ratio) mixed with loading dye, such that the minimum quantity of siRNA in each well was 1µg (Fig. 2). The band intensity was measured using BIORAD, Molecular Imager Chemi Doc XRS. The results showed that the DTX-PAMAM conjugate with 3 DTX molecules per dendrimer was able to form complex even at 5:1 N/P ratio (Fig 2). The particle size and zeta potential of this multifunctional system is shown below (Table 2). As can be seen from Table 2, the size was in the range of ~150nm and with a uniform size as indicated by the low polydispersity index. The decrease in zeta potential after siRNA complexation confirmed the formation of the multifunctional nanotherapeutic system.

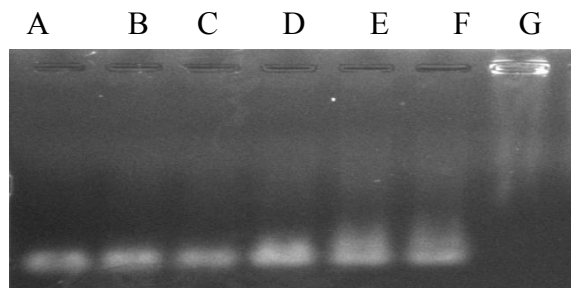
Table 2: Size and Zeta potential of dendrimer, dendrimer-DTX and dendrimer-DTX-siRNA complex.

Parameter	PAMAM	PAMAM-DTX	PAMAM-DTX-siRNA
Size (nm)	118.08±0.8	157.9±2.5	142.4±7.8
Zeta Potential (mV)	41.9±1.4	33.1±1.1	12.7±3.4
Polydispersity Index	0.125	0.124	0.158



- A- DTX-G4PAMAM CONJ. – siRNA (5:1)
- B- DTX-G4PAMAM CONJ. – siRNA (10:1)
- C- DTX-G4PAMAM CONJ. – siRNA (20:1)
- D- siRNA alone

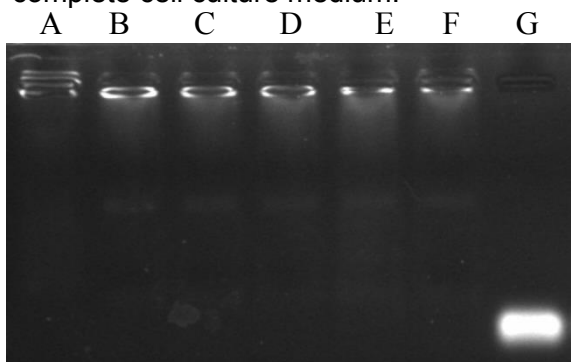
Fig 2: Agarose gel electrophoresis to confirm the complex formation between dendrimer-DTX conjugate and siRNA at various Nitrogen/Phosphorus (N/P) ratios (BIORAD, Molecular Imager Chemi Doc XRS).



- A- siRNA alone
- B- DTX-PAMAM-siRNA with dextran sulfate (5:1 w/w)
- C- DTX-PAMAM-siRNA with dextran sulfate (10:1 w/w)
- D- DTX-PAMAM-siRNA with dextran sulfate (20:1 w/w)
- E- DTX-PAMAM-siRNA with dextran sulfate (40:1 w/w)
- F- DTX-PAMAM-siRNA with dextran sulfate (80:1 w/w)
- G- DTX-PAMAM-siRNA (10:1 N/P)

Fig 3: Agarose gel (1%) electrophoresis of PAMAM-DTX-siRNA at different N/P ratios; effect of dextran sulfate.

The formation of the complex was further confirmed by incubating the complexes with dextran sulfate (a negatively charged macromolecule that displaces siRNA from dendrimer). As shown in Fig 3, the siRNA was released from the complex on incubating with dextran sulfate. We then tested the stability of the complex in presence of cell culture medium. Briefly, PAMAM-DTX-siRNA complex was incubated with the culture media for different time intervals up to 12 hours and then gel electrophoresis was performed to observe the stability of the complex. As shown in Fig 4 the complex remained intact and stable even after 12 hours of incubation with the complete cell culture medium.



- A- DTX-PAMAM-siRNA control (5:1)
- B- DTX-PAMAM-siRNA in culture media (1 h)
- C- DTX-PAMAM-siRNA in culture media (2 h)
- D- DTX-PAMAM-siRNA in culture media (4 h)
- E- DTX-PAMAM-siRNA in culture media (6 h)
- F- DTX-PAMAM-siRNA in culture media (12h)
- G- siRNA only

Fig 4: Agarose gel (1%) electrophoresis of PAMAM-DTX-siRNA at different N/P ratios effect of incubation time with culture media containing serum. Culture media was added in 1:1 ratio.

Release studies

To test the release of DTX from the multifunctional system the drug release studies were performed in PBS and in cell culture medium. Briefly, 2.5 mg of PAMAM-DTX conjugate was dissolved in 50 μ L of methanol and was added directly to 5 mL of respective media in a glass vial in triplicate. At different time points 100 μ L of the sample was withdrawn and after each sample withdrawal 100 μ L fresh media was added. 100 μ L sample withdrawn was treated with 1 mL of ethyl acetate for 15-20 minutes with constant vortexing to extract the released DTX. 500 μ L of the supernatant was then taken in another eppendorf tube and the ethyl acetate was evaporated under nitrogen gas. The dried residue was then reconstituted with 1 mL of methanol and analyzed for drug content by HPLC (Acetonitrile/ Water 1:1, Run time 10 minutes, RT 7.98 minutes, sample volume injected 20 μ L). The

results showed that around 50% drug was released in 24 hrs (Fig 5) and the release was not significantly different between cell culture medium and PBS. The results indicate the DTX is released slowly over a period of 24 hrs.

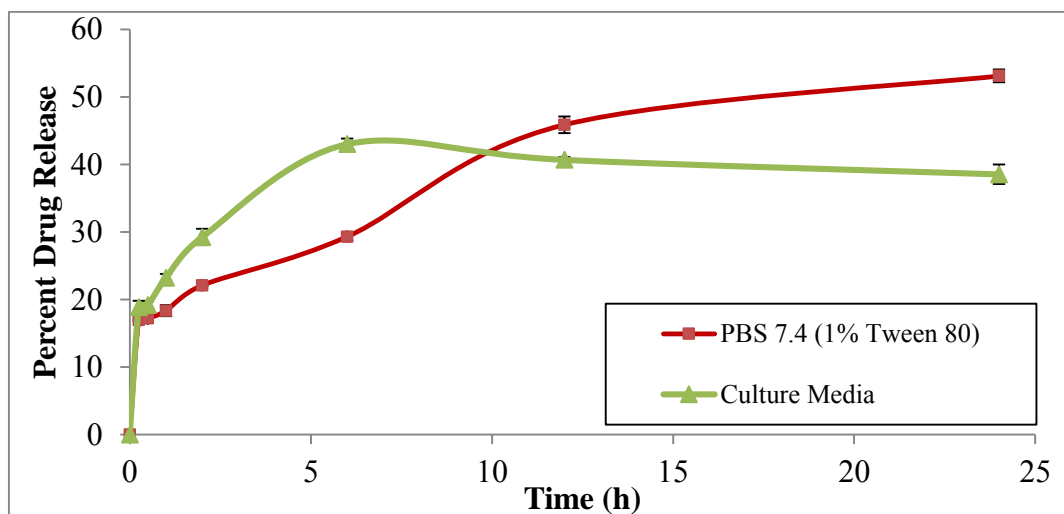


Fig 5: *In vitro* release studies in Phosphate buffer saline (PBS) 7.4 (with 1% Tween 80 to maintain sink conditions) and cell culture Media (DMEMDMEM + 10% FBS + 1% Streptomycin Penicillin). Values represents Mean \pm SD (n=3). DTX release was measured by HPLC.

Specific Aim 3: Test the activity of nanotherapeutic system in PC cells

During the last evaluation period, we tested the cell uptake of siRNA-dendrimer complex and also measured the gene silencing ability of the complex in drug sensitive PC3 cells. We found that the complex was able to reduce the eIF4e levels in drug sensitive PC3 cells. During the current evaluation period, our goal was to test the in-vitro efficacy of the multifunctional therapeutic system in drug sensitive and drug resistant PC3 cells. The IC₅₀ values of different formulations were determined in drug sensitive PC3 and drug resistant prostate cancer (PC3dR) cells. The cells were seeded in a 96 well plate at cell density of 10000 cells/well and cultured overnight. The following day, cells were treated with serial dilutions of different formulations so that the DTX (10nM to 500 nM) and/or siRNA concentration (6ng/ml) for 48 hrs. At the end of treatment, the drug solution was removed and the cell viability was tested by MTT assay (Table 3).

Table 3: IC₅₀ values of DTX in drug sensitive and resistant PC3 cells

Treatment	IC ₅₀ Values	
	PC3 (drug sensitive cells)	PC3dR (drug resistant cells)
DTX	200 \pm 2.3nM	500 \pm 0.72nM
DTX-Dendrimer	50 \pm 0.34nM	100 \pm 6.49nM
DTX-Dendrimer-siRNA	50 \pm 1.83nM	25 \pm 0.82nM

The IC₅₀ value of DTX in resistant PC3 cells was 2.5 fold higher than drug sensitive PC3 cells. Conjugation to the dendrimer decreased the IC₅₀ value of DTX by increasing the drug's water solubility and cell uptake. However there was still 2 fold difference in the IC₅₀ value between drug resistant and drug sensitive PC3 cells. On combining with eIF4E siRNA the IC₅₀ value of DTX was reduced by half in drug sensitive PC3 cells, while there was no change in IC₅₀ value in drug sensitive PC3 cells. On the other hand for free siRNA or siRNA-dendrimer IC₅₀ value were outside the concentration range tested (i.e. >1000nM). Overall the results show that combination therapeutic system can reverse the drug resistance in prostate cancer cells.

To measure the levels of eIF43 and related downstream protein, western blot was performed. After 24 hours of treatment, the culture media was removed and cells were harvested, washed with PBS and lysed using lysis buffer. Protein estimation in cell lysates was carried out using a Pierce® BCA Protein Assay Kit (Thermo Scientific, USA). An equal amount of protein from cell lysates was loaded in the gel and subjected to SDS-PAGE (10% Acrylamide Gel) followed by immunoblotting. The nitrocellulose membrane was blocked with 5% skimmed milk for 1 h in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) at room temperature and probed for total eIF4E, tubulin, and cMyc followed by treatment with corresponding antibodies and ECL detection. HRP

conjugated secondary anti-mouse and rabbit antibodies was used. As shown in Fig 6, the eIF4E levels were significantly elevated in the DTX resistant cells. The treatment with dendrimer-siRNA significantly reduced the eIF4E levels in both sensitive and resistant cell lines. The DTX-dendrimer-siRNA complex reduced the eIF4E levels in resistant cells relatively more compared to the drug sensitive prostate cancer cells. Although the DTX-dendrimer-siRNA complex reduced the eIF4E levels, the levels were relatively high compared to DTX-siRNA complex. This may be due to the effect of DTX on siRNA loading, cell uptake and/or its release from the complex. We also measured the levels of Myc, a transcription factor that is controlled by eIF4E. Myc is a proto-oncogene that is upregulated in many cancers including prostate cancer⁶. As can be seen in Fig 7, Myc levels are elevated in both drug sensitive and resistant PC3 cells. Treatment with eIF4E siRNA-dendrimer complex and eIF4E siRNA-DTX-dendrimer conjugate significantly decreased the levels of Myc. The decrease was similar for both the complexes. The Myc may be more sensitive to the inhibitory effect on eIF4E levels. Further studies are required to optimize the cell uptake and release of the multifunctional system and also delineate the mechanism of cell death in PC3 cells. Nevertheless, the results demonstrate the potential of the multifunctional nanotherapeutic system to overcome drug resistance in prostate cancer.

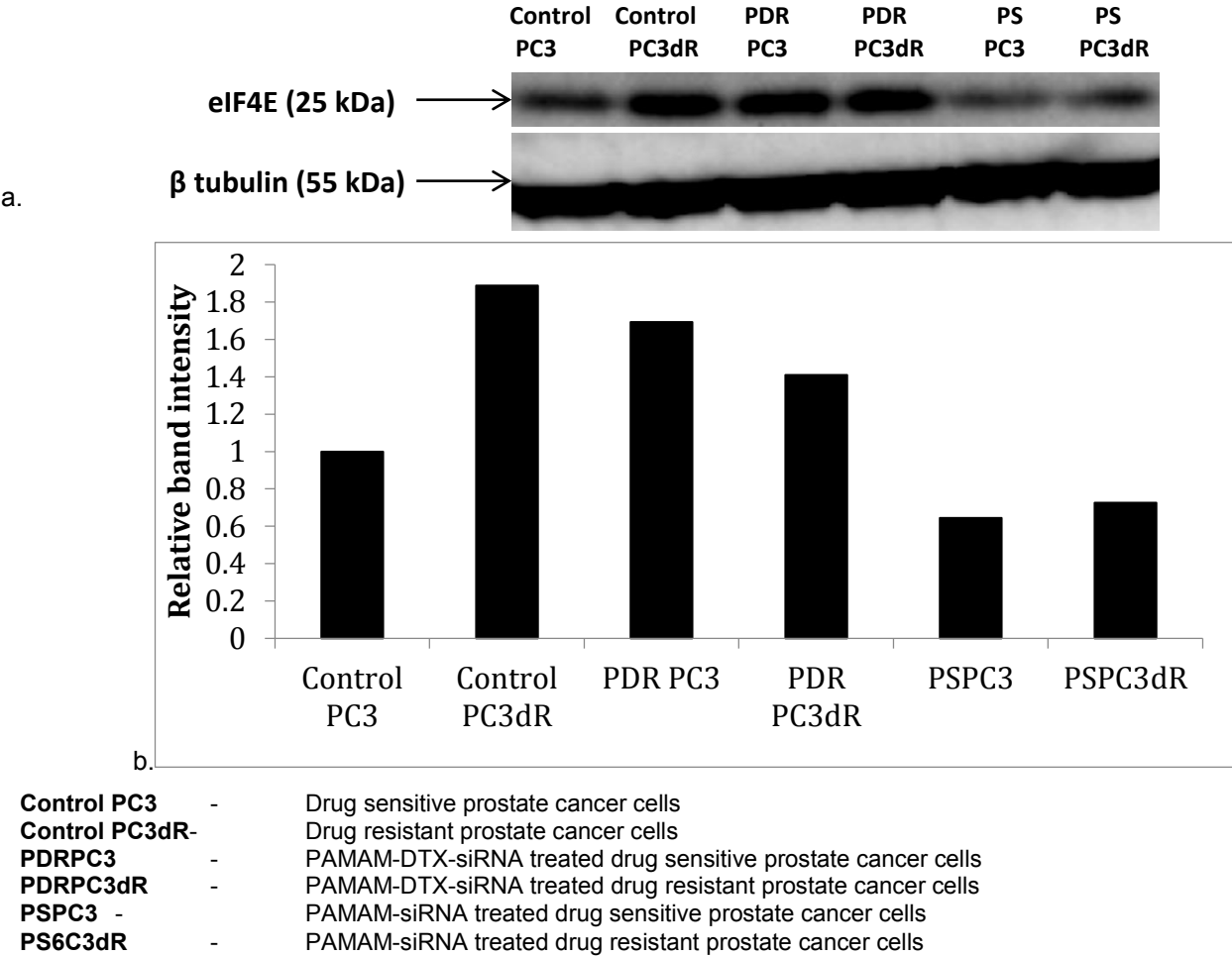
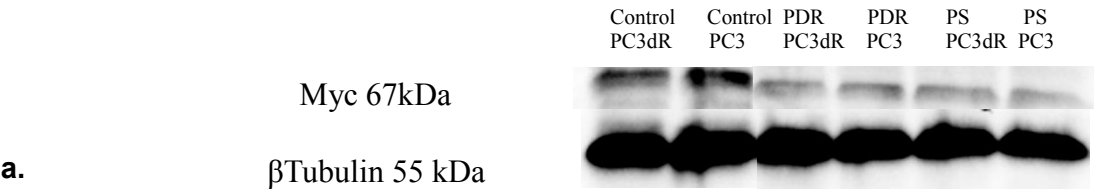
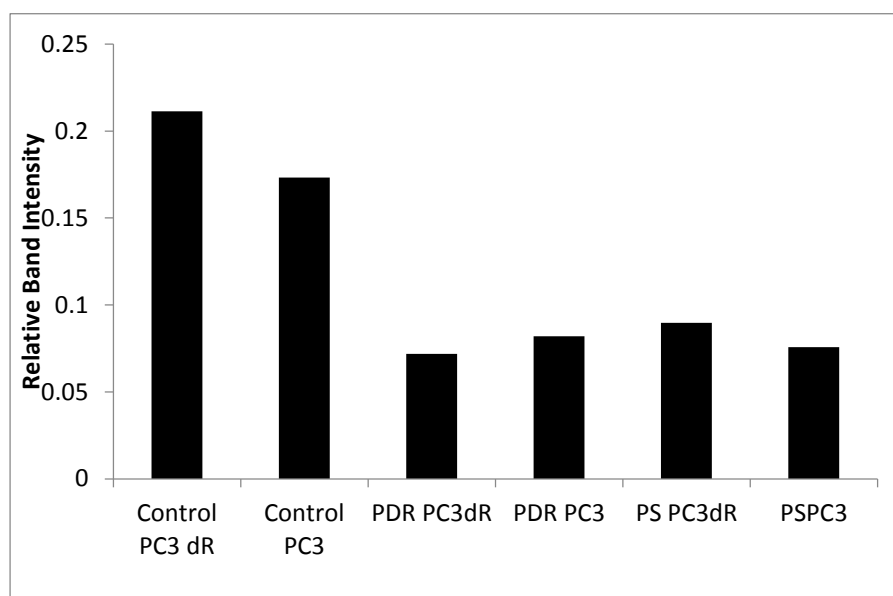


Fig 6: Band (a) and band intensity (b) of eIF4E for different treatment groups.





b.

Control PC3	-	Drug sensitive prostate cancer cells
Control PC3dR-		Drug resistant prostate cancer cells
PDRPC3	-	PAMAM-DTX-siRNA treated drug sensitive prostate cancer cells
PDRPC3dR	-	PAMAM-DTX-siRNA treated drug resistant prostate cancer cells
PSPC3	-	PAMAM-siRNA treated drug sensitive prostate cancer cells
PSPC3dR	-	PAMAM-siRNA treated drug resistant prostate cancer cells

Fig 7: Band (a) and band intensity (b) of Myc for different treatment groups.

Key Research Accomplishments

- A drug resistant PC3 cell was developed that overexpressed eIF4E.
- A multifunctional nanotherapeutic system was developed by conjugating DTX to dendrimer and then using the conjugate to complex eIF4E siRNA.
- The multifunctional system reduced the IC₅₀ value of DTX in resistant PC3 cells and reversed drug resistance.
- The siRNA reduced the levels of eIF4E and Myc in prostate cancer cells.

Reportable Outcome

- Presented poster on titled “Multi-functional Dendrimer Based Nanotherapeutic System for Prostate Cancer” in 40th Annual Meeting and Exposition of the Controlled Release Society, 21-24 July, 2013 at Honolulu, Hawaii, USA.
- Given there are no commercial drug resistant PC3 cells, we faced significant challenge in developing the DTX resistant PC3 cells. The cells were procured from a leading prostate cancer research group from Canada. The treatment protocol takes 9 months to develop DTX resistant PC3 cells. One of the significant outcome is the development of the DTX resistant cells which can be used for studying therapeutic approaches for drug resistant prostate cancer.
- The data generated from this project will be used to further optimize the nanotherapeutic system and study the mechanism of cell death in drug resistant prostate cancer. In particular there were differences in the eIF4E levels between DTX-dendrimer-siRNA and dendrimer-siRNA complexes. This needs to be clarified by studying the effect of DTX on siRNA loading, cell uptake and release in the cell. Once this data is validated we plan to publish the findings. Further the results will serve as a preliminary data to apply for a larger grant to DoD and NIH to test the in-vivo efficacy and pharmacokinetics of this novel combination therapy.
- The graduate student who worked in this project was trained in prostate cancer as well as cell culture. The student has graduated and has taken up research scientist position with Allergan Inc.
- The personnel who received support from this grant includes the following; Preety Sahdev (graduate student), Omathanu Perumal (PI), Xiangming Guan (Co-I), Radhey Kaushik (Co-I).

Conclusions

The goal of this project was to develop a multifunctional nanotherapeutic system for drug resistant prostate cancer. We successfully developed a method to prepare multifunctional therapeutic system to simultaneously deliver a chemotherapeutic drug and a siRNA. The proof of concept from this study demonstrates the potential to overcome drug resistance by silencing eIF4E. There are significant challenges in developing drug resistant prostate cancer cells and we successfully developed the resistant cell line through this project. Although the multifunctional nanotherapeutic system lowered the IC₅₀ value of DTX in drug resistant cells, the study was inconclusive regarding the mechanism of cell death. To this end the multifunctional delivery system has to be further optimized for drug loading and release. Detailed mechanistic studies are required to understand the mechanism of cell death to validate the findings from this study. The successful completion of these additional in-vitro studies will lead to the further preclinical and clinical development of this novel combinatorial therapy for drug resistant prostate cancer cells. In addition the findings from this study can be extended to the combinatorial therapy involving chemotherapeutic agents and gene silencing in prostate as well as other cancers.

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